## REMARKS

Following entry of the foregoing amendments, claims 11-16 will be pending. Claim 13 has been amended. Support for the amended claim may be found throughout the specification including in the original claims, for example at page 57, lines 20-21, and in the sequence listing. No new matter is added by these amendments.

**Rejections Under 35 U.S.C. § 101**

The Examiner rejected claims 11-16 under 35 U.S.C. § 101, because the claimed invention allegedly "is not supported by either a specific asserted utility or a well established utility." Office Action at pages 2-3 . The Board of Appeals and Interferences sustained the rejection on the basis that "the Specification...never connects ... [SEQ ID NO:48411] to any particular or specific utility." *See Decision on Appeal* 2008-2230 September 24, 2008, at pages 5-6.

Applicants respectfully request reconsideration in light of the additional evidence and arguments provided.

The specification as filed discloses that SEQ ID NO: 48411 has significant homology to the sequence disclosed in NCBI record CAA18117, which is associated with the sequence identifier "3046693" (*i.e.*, gi:3046693).[1] A review of that record indicates that the source of the sequence (DBSOURCE) is "embl accession AL022140.1". An examination of the record AL022140 indicates that the sequence associated with the identifiers CAA18117 and gi:3046693 has "strong similarity to ES43 protein..." of Barley (*H. vulgare*). Applicants respectfully submit that a skilled artisan would understand that barley ES43 protein is recognized in the art as a barley steroid hormone receptor. *See, e.g.*, NCBI record X77575 and Speulman and Salamini, *A barley cDNA clone with homology to the DNA-binding domain of the steroid receptor*, Plant Sci. 106, 91-98 (1995).[2]

---

[1] A copy of the record associated with CAA18117 gi:3046693 is provided for the Examiner's consideration.

[2] A copy of both record X77575 and Plant Sci. 106, 91-98 (1995) are provided for the Examiner's consideration.

In *In re Fisher*, the Federal Circuit reiterated that the "basic *quid pro quo* contemplated by the Constitution and the Congress for granting a patent monopoly is the benefit derived from the public from an invention with *substantial utility*." *In re Fisher*, 421 F.3d 1365, 1371 (Fed. Cir. 2005) (citing *Brenner v. Manson*, 383 U.S. at 534-35, 1966) (emphasis in original). The Court noted that since "*Brenner* our predecessor court, the Court of Customs and Patent Appeals, and this court have required a claimed invention to have a specific and substantial utility to satisfy § 101." *Id.* Furthermore, an invention need only provide one identifiable benefit to satisfy 35 U.S.C. § 101. *See Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 958 (Fed. Cir. 1983) ("when a properly claimed invention meets at least one stated objective, utility under section 101 is clearly shown").

Although the Supreme Court has not defined the meaning of the terms "specific" and "substantial", the Federal Circuit has identified a framework for the kind of disclosure an application could contain to establish a specific and substantial utility. *In re Fisher*, 421 F.3d at 1371. First, the Court indicated that, to provide a substantial utility, the specification should disclose a utility such that "one skilled in the art can use a claimed discovery in a manner which provides some *immediate benefit to the public*." *Id.* (emphasis in original). Second, a specific utility can be disclosed by discussing "a use which is not so vague as to be meaningless," that is that the claimed invention "can be used to provide a well-defined and particular benefit to the public." *Id.*

In view of the foregoing, a skilled artisan, at the time of the invention, would recognize that the nucleotide sequence of SEQ ID NO: 48411 can be employed to, among other things, be used to identify putative plant receptors, which influence a variety of processes through regulation of transcription. In other words, SEQ ID NO: 48411 has at least one utility specific to it and not generally applicable to any nucleic acid. Therefore, Applicants have disclosed at least one specific, substantial, and credible utility for the claimed invention that provides an immediate benefit to the public, and therefore the claimed invention meets the utility test set forth in *In re Fisher*. Thus, Applicants respectfully request that the Examiner withdraw the rejection of claims 11-16 under 35 U.S.C. § 101.

**Rejections Under 35 U.S.C. § 112 first paragraph**

**A. The Claimed Invention is Supported by a Statutory Utility**

The Examiner rejected claims 11-16 under 35 U.S.C. § 112, first paragraph, because, allegedly, "the claimed invention is not supported by either a specific asserted utility or a well established utility... one skilled in the art clearly would not know how to use the claimed invention." Office Action at pages 3-4. Applicants respectfully disagree with the rejection, and submit that this rejection has been overcome by the arguments set forth above with respect to the rejection under 35 U.S.C. § 101. In other words, Applicants respectfully submit that, since the claimed invention has at least one specific, substantial, and credible utility, the rejection of claims 11-16 with respect to the enablement requirement of 35 U.S.C. § 112, first paragraph must be withdrawn.

**B. The Claimed Invention is Supported by an Adequate Written Description.**

Applicants respectfully note the Decision of the Board of Appeals and Interferences reversing the rejection of clams 11-15 under 35 U.S.C. § 112 for allegedly lacking an adequate written description. *See Decision on Appeal* 2008-2230 September 24, 2008, at pages 6-7.

**Rejections Under 35 U.S.C. § 102**

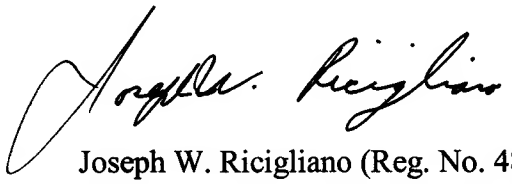
The Examiner rejects claim 13 under 35 U.S.C. § 102(b) over Maharias *et al.* (Gen EMBL Accession No. AQ451805). The Board of Appeals and Interferences affirmed the decision on the basis that the 21 nucleotide fragment present in Maharias reads on "about 30" nucleotides.

Applicants respectfully disagree. In order to advance prosecution, however, Applicants have amended claim 13 to delete the term "about." In view of the foregoing, Applicants respectfully request reconsideration and withdrawal of the rejection.

**CONCLUSION**

In view of the above, each of the presently pending claims is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue. The Examiner is encouraged to contact the undersigned at (202) 942-5000 should any additional information be necessary for allowance.

Respectfully submitted,



Joseph W. Ricigliano (Reg. No. 48, 511)
Holly Logue Prutz (Reg.No. 47,755)
David R. Marsh (Reg. No. 41,408)

Date: November 19, 2008

ARNOLD & PORTER LLP
Attn: IP Docketing
555 12th Street, N.W.
Washington, D.C. 20004
(202) 942-5000 telephone
(202) 942-5999 facsimile

1: CAA18117. Reports receptor like pro...[gi:3046693]

BLink, Conserved Domains, Links

Comment Features Sequence

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DEFINITION  receptor like protein (fragment) [Arabidopsis thaliana].
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DBSOURCE    embl accession AL022140.1
KEYWORDS    .
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REFERENCE   1  (residues 1 to 201)
  AUTHORS   Bevan,M., Wedler,H., Wambutt,R., Bancroft,I., Mewes,H.W., Lemcke,K.
            and Mayer,K.F.X.
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 201)
  AUTHORS   EU Arabidopsis sequencing,project.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-MAR-2000) MIPS, at the Max-Planck-Institut fuer
            Biochemie, Am Klopferspitz 18a, D-82152 Martinsried, FRG, E-mail:
            lemcke@mips.biochem.mpg.de,mayer@mips.biochem.mpg.de Project
            Coordinator: Mike Bevan, Molecular Genetics Department, Cambridge
            Laboratory, John Innes Centre, Colney Lane, NR4 7UJ Norwich, UK,
            E-mail: michael.bevan@bbsrc.ac.uk
COMMENT     Information on performance of analysis and a more detailed
            annotation of this entry and other sequences of chromosomes 3, 4
            and 5 can be viewed at: http://www.mips.biochem.mpg.de/proj/thal/.
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1: X77575. Reports H.vulgare (Dbg576...[gi:482918] Links

Features Sequence

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DEFINITION  H.vulgare (Dbg576) ES43 mRNA.
ACCESSION   X77575
VERSION     X77575.1  GI:482918
KEYWORDS    ES43 gene.
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            clade; Pooideae; Triticeae; Hordeum.
REFERENCE   1
  AUTHORS   Speulman,E. and Salamini,F.
  TITLE     A barley cDNA clone with homology to the DNA-binding domain of the
            steriod hormone receptors
  JOURNAL   Plant Sci. 106, 91-98 (1995)
REFERENCE   2  (bases 1 to 1058)
  AUTHORS   Speulman,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-1994) E. Speulman, Max-Planck-Inst. f.
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PLANT SCIENCE

# A barley cDNA clone with homology to the DNA-binding domain of the steroid hormone receptors ☆

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**Abstract**

The steroid hormone receptors are members of a superfamily of nuclear receptors that regulate gene expression in response to a ligand in mammals and insects. A cDNA library from barley (*Hordeum vulgare*) was screened with highly degenerated oligonucleotides corresponding to 11 and 6 amino acids of the highly conserved DNA-binding domain of the steroid hormone receptors. A full length cDNA clone (ES43) was isolated. The sequence of ES43 shows homology to the DNA-binding domain of the estrogen receptor, including three amino acids ($Glu^{191}$, $Gly^{192}$ and $Ala^{195}$) at conserved positions, which determine the binding specificity of the estrogen receptor to its hormone response element. Additional homologies concerning critical amino acid positions with all members of the nuclear receptor superfamily are also evident.

*Keywords:* *Hordeum vulgare*; Steroid hormone receptors; DNA-binding domain; Zinc-finger

## 1. Introduction

Steroid hormones, plant sterols, and gibberellins are products of the terpenoid pathway with close structural relationships. The steroid hormones regulate events involved in development and differentiation in mammals and insects. They mediate their effect through binding to specific high affinity receptors [1] which belong to a superfamily of nuclear receptors for the steroid and thyroid hormones, vitamin $D_3$ and retinoic acid. Gene expression in response to a ligand is modulated by the receptor through binding to specific hormone response elements (HREs) in the promoter of target genes [1,2]. To date, no homologs of this class of transcription factors have been reported isolated from plants.

All receptors cloned so far, share a similar structure: a variable N-terminal region, a highly conserved cysteine rich domain and an only partly conserved C-terminal region, where the ligand

☆ The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X77575 H.vulgare (Dbg576) ES43 mRNA.

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*SSDI* 0168-9452(95)04072-3

binding domain is located [3,4]. The highly conserved DNA-binding domain, corresponds to the cysteine rich region, with cysteine residues arranged into two zinc fingers, each of them with a zinc ion tetrahedrally coordinated by four cysteines [5]. The two zinc finger motifs have separate functions. The N-terminal finger serves as the primary DNA-recognition unit and three distinct amino acids are responsible for the specific binding of the receptors to their response element [6,7]. The steroid and thyroid receptors bind as dimers to their hormone response element. The C-terminal zinc finger motif is responsible for these protein interactions between two receptor molecules [8].

Steroid hormone receptors have been shown to function as hormone dependent inducers of gene expression in yeast and plants [9–11]. Expression of the genes encoding the human estrogen and glucocorticoid receptor in yeast and tobacco results in transactivation of a hybrid promoter carrying HRE sequences in a hormone specific dependent manner [9,10]. The molecular mechanisms underlying this activation must, therefore, be very well conserved across eukaryotes.

Gibberellins regulate several developmental and differentiation processes in plants. Like other plant hormones, they may act through tissue specific receptors. The structural relationship between steroid hormones, vitamin D, retinoic acid and gibberellins might imply a relationship between the receptors. This relationship suggested an approach to isolate steroid hormone receptor homologs from plants with the aim of isolating nuclear receptors, and potentially plant hormone receptors.

In this paper we describe the isolation of a barley cDNA clone that shares homology with the DNA-binding domain of the nuclear hormone receptors.

## 2. Material and methods

### 2.1. Plant material

A GA-sensitive barley dwarf mutant, Dornburg 576 (*dbg576*), kindly provided by Dr W. Hentrich, was grown in the greenhouse under 16 h light/8 h dark conditions at 18°C day/ 12°C night, with a light intensity of around 270 $\mu E \cdot m^{-2} \cdot s^{-1}$.

### 2.2. RNA isolation and analysis

Poly(A)$^+$RNA was isolated from 2-month-old *dbg576* leaves, as described in Bartels and Thompson [12]. Total RNA was isolated from 2-month-old *dbg576* tissue, according to Koes et al. [13]. For Northern analysis, 2 $\mu$g of poly(A)$^+$RNA or 10 $\mu$g of total RNA was separated on a 1.3% formaldehyde-agarose gel [14], and blotted onto a GeneScreen Plus membrane (NEN, duPont), according to the manufacturer's instructions. The filter was hybridized for 24 h at 58°C in 1 M NaCl, 10% dextran sulfate (Pharmacia), 1% SDS, 200 $\mu$g/ml denatured herring sperm DNA, and $^{32}$P-labelled *NotI-SalI* ES43 digested insert. Labelling was according to Feinberg and Vogelstein [15]. The filter was washed for 2 × 5 min in 2× SSC at room temperature and for 2 × 30 min in 2× SSC, 1% SDS at 58°C.

### 2.3. Degenerated oligonucleotides

Degenerated oligonucleotides were purified over NAP™-10 columns (Pharmacia). The oligonucleotide sequences were, for oligo A, ACI TG(C/T) GA(A/G) GGI TG(C/T) AA(A/G) GGI TT(T/C) TT(T/C) CGI (A/C)G, corresponding to the amino acids TCEGCKGFFKR, and for oligo B, AC(AGGC/T) TG(C/T) GA(A/G) GG(AGGC/T) TG (C/T) AAG, corresponding to the amino acids TCEGCK.

### 2.4. Construction and screening of a cDNA library

A cDNA library was constructed from 2 $\mu$g poly(A)$^+$RNA of $GA_3$-treated dwarf mutant leaves, using the Superscript™ system (BRL, Life Technologies), and competent cells of the bacterial strain DH5$\alpha$ (BRL, Life Technologies), according to the manufacturers instructions. Transformants were plated onto YT plates containing 100 $\mu$g/ml of ampicillin and, after overnight incubation at 37°C, transferred to Colony/Plaque Screen™ discs (NEN, duPONT). Around 70 000 colonies were hybridized with oligo A or B. The oligonucleotides were 5′-end labelled according to Sambrook et al. [14], using 5000 Ci/mmol [$\gamma$-$^{32}$P]ATP (Amersham). Filters were prehybridized in 1 M NaCl, 10% dextran sulfate, 1% SDS for at least 12 h at 47°C for oligo A and at 33°C for oligo B. Approximately $10^6$ counts/min (1 ng) of la-

```
1     GAGCTCGCTCCCATTCCCATCCCCATCCCATCCCATCCCACCCCCTCCCC
51    CTCGTCGGAGCCGCGCCGCCGCCGCCTCCCAATCCGCCGCCGCCGCCGCC
101   CATGTCAAAGACGCCGGGGAAGTCGCCGCGGACGCCGCGCCGGACCCTGG
        M  S  K  T  P  G  K  S  P  R  T  P  R  R  T  L  E      17
151   AGTCCTACACCATCAAGGGATCCGACGGCGTCATCCGCCCTGGAGACTCT
        S  Y  T  I  K  G  S  D  G  V  I  R  P  G  D  S        33
201   GTGCTCATGAAGGCGCCCGACTCATCAAAGCCTCCATATGTGGCCAAGAT
        V  L  M  K  A  P  D  S  S  K  P  P  Y  V  A  K  I     50
251   TGAGGAAATCGAGGCAGCAGGACCTCGGGGCGCAAATGTCAAGGTTAAAG
        E  E  I  E  A  A  G  P  R  G  A  N  V  K  V  K  V     67
301   TGCGGTGGTACTACCGGCCAGAAGAGTCTATTGGTGGGCGGAGGCCATTC
        R  W  Y  Y  R  P  E  E  S  I  G  G  R  R  P  F        83
351   CATTGCGAAAAGGAGGTGTTCCTCTCCGACCACCAAGATGTGCAGAGTGC
        H  C  E  K  E  V  F  L  S  D  H  Q  D  V  Q  S  A     100
401   TGACACCATAGAGTGCAAGTGCAATGTCTACAGCTTCCGGGACTACACCA
        D  T  I  E  C  K  C  N  V  Y  S  F  R  D  Y  T  K     117
451   AGCTTGCTGCTGTCAATCCTGAGGACTATTTTTGCCGCTTCGAGTACCAG
        L  A  A  V  N  P  E  D  Y  F  C  R  F  E  Y  Q        133
501   TCAATCACAGGCAGCTTTGTGCCTGACCGCATAGCTGTGTTTTGCAAGTG
        S  I  T  G  S  F  V  P  D  R  I  A  V  F  C  K  C     150
551   TGAGATGCCATACAACCCTGACGATCTTATGATCCAGTGTGAGGAATGCT
        E  M  P  Y  N  P  D  D  L  M  I  Q  C  E  E  C  S     167
601   CTGATTGGTTTCACCCTGCTTGTATTGGAAAGACCATAAAAGAAGCAAAG
        D  W  F  H  P  A  C  I  G  K  T  I  K  E  A  K        183
651   AAGCTTGAGAATTTTACATGCGAAGGTTGCGTTGCTGAAAATGGAAATGC
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701   GAATGGAGTCAAGAATGAAAATTCGCATGAATCTACAGGCGAGTCAGATG
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851   GCTGTTTCCAGTTTGATGTAGAACGAACCTGTATTGGGAGTCTCTTCTAG
901   AGTCTGAGTCTGGCAGTCGGCCAGTTTGCGACTTTGCATATCCATGTGGA
951   CCTGTCCTGTTAGAGGGAGTCATTATTTTTCTCTCTTCTTTTTTCCTTCG
1001  CCCCCCCTTTGTGTCGACTTGGACTCTGGTGAGTGAGCTTAAACGTCCTT
1051  CCTTGACT(A)x
```
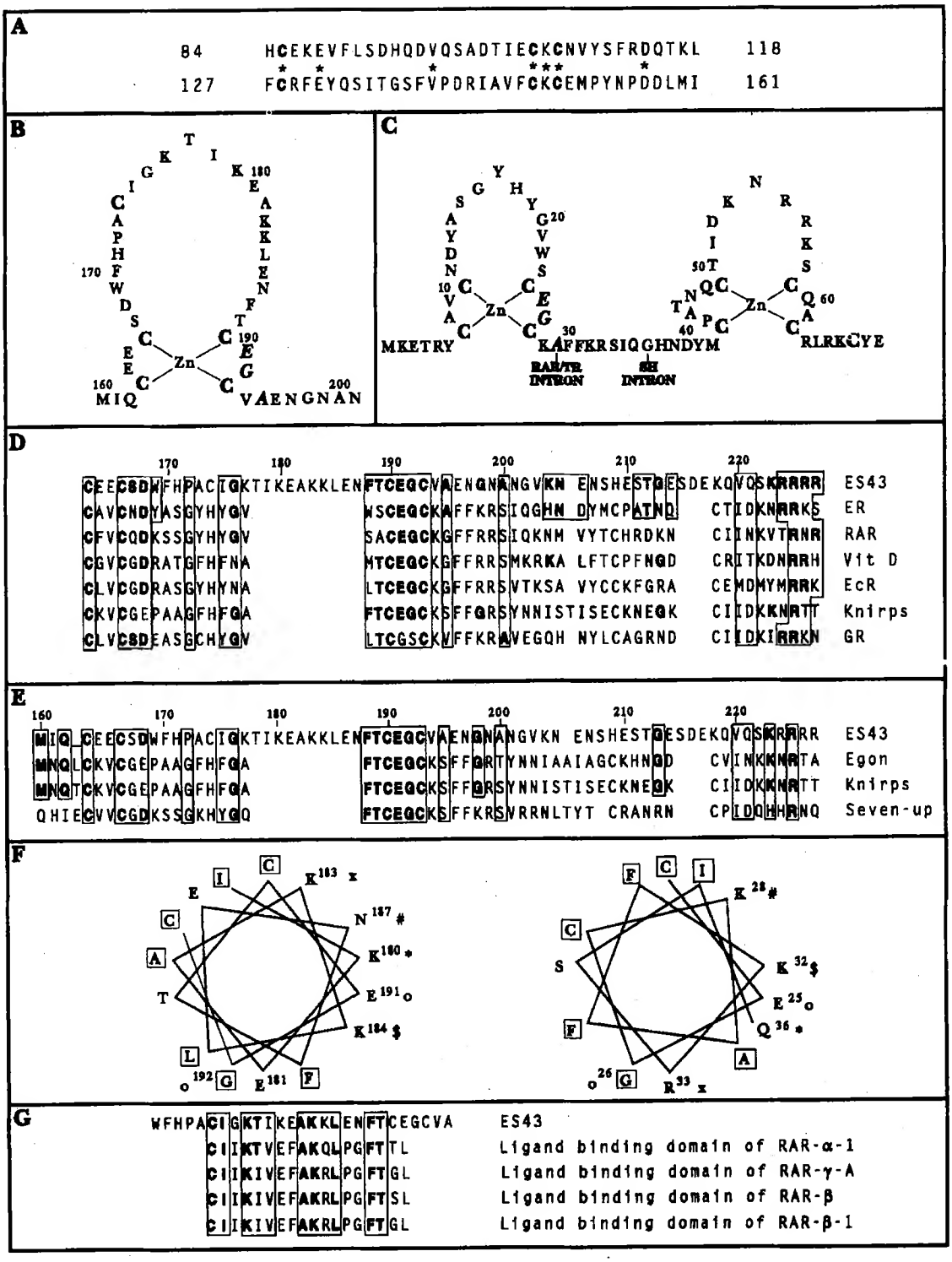

Fig. 1. Nucleotide and predicted amino acid sequence of ES43. Nucleotide sequence of full length ES43 is presented in the 5′ to 3′ orientation with numbering at the left side of the sequence. The translational open reading frame is shown below the nucleotide sequence with numbering at the right side of the sequence. Cysteine residues are shown in bold. The potential zinc-finger is underlined. The position of the poly-A-tail is marked as $(A)_x$.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127 FCRFEYQSITGSFVPDRIAVFCKCEMPYNPDDLMI 161
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RLRKCYE
RAR/TR INTRON
RH INTRON
D
ES43
ER
RAR
Vit D
EcR
Knirps
GR
E
ES43
Egon
Knirps
Seven-up
F
G
WFHPACIGKTIKEAKKLENFTCEGCVA ES43
CIIKTVEFAKQLPGFTTL Ligand binding domain of RAR-α-1
CIIKIVEFAKRLPGFTGL Ligand binding domain of RAR-γ-A
CIIKIVEFAKRLPGFTSL Ligand binding domain of RAR-β
CIIKIVEFAKRLPGFTGL Ligand binding domain of RAR-β-1

belled oligonucleotide per ml of hybridization solution were hybridized for 48 h at 47°C for oligo A and at 33°C for oligo B. Filters were washed for 3 × 5 min in 6× SSC, 0.05% sodium-pyrophosphate and for 15 min in 6× SSC, 0.05% sodium-pyrophosphate at, respectively 47°C and 33°C. Exposure of the filters to XAR films (KODAK) was for 48 h at −70°C.

### 2.5. DNA sequencing

The DNA sequences were determined by the chain termination method on double stranded templates [16] using the $^{T7}$Sequencing kit (Pharmacia). The cDNA clones were sequenced with sequence specific oligonucleotides in forward and reverse mode. Sequence assembly and analysis were done with programs from the GCG software package 7.1 [17].

## 3. Results

### 3.1. Isolation and sequence of ES43

Around 70 000 colonies of a barley cDNA library were screened with the degenerated oligonucleotides A and B, which correspond to the most conserved region of the DNA-binding domain of the nuclear hormone receptors. The DNA-binding domain of the nuclear hormone receptors represents the most conserved region and was therefore preferred to the relatively low conserved ligand binding domain, which was expected not to be evolutionary conserved. One cDNA clone was found positive with oligo A, but after sequence analysis revealed a potential zinc finger not related to the DNA binding domain of the steroid hormone receptors. Of two cDNA clones found positive with oligo B one, designated ES43, contained the oligonucleotide sequence used for screening and a potential zinc finger motif.

Sequence analysis of ES43 revealed an insert of 1058 bp (Fig. 1). The deduced amino acid sequence is 227 amino acids long, which would correspond to a molecular weight of 26 kDa. The protein is relatively rich in cysteines (11 residues). Six cysteines are positioned in the first part of the protein, and seem to be located at fixed positions in a repeat (Fig. 2A). Homologies between this part of ES43 and proteins in the databases were restricted to only small portions of the protein and concerned the conserved positions of cysteines in proteins containing cysteine rich regions. Five cysteine residues are located in the C-terminal region and encode a putative zinc-finger between amino acids 160 and 200 (Fig. 2B). The region covering oligonucleotide B (TCEGCK), responsible for specific DNA-binding of the estrogen receptor to its hormone response element (HRE), is located between amino acids 189 and 194. A po-

Fig. 2. Features of ES43 and the nuclear hormone receptors. (A) Sequence alignment of the 5′ part of ES43, between amino acids 84 and 118 and between 127 and 161. Conserved cysteine residues are shown in bold. Homologous amino acids are indicated with an asterisk. (B) Potential zinc-finger of ES43 between amino acids 160 and 201. The amino acid residues Glu$^{191}$, Gly$^{192}$ and Ala$^{195}$, determine binding specificity of the estrogen receptor and are shown in bold-italic. (C) The DNA-binding domain of the estrogen receptor represented as two zinc-fingers. The amino acid residues Glu$^{25}$, Gly$^{26}$ and Ala$^{29}$ are shown in bold-italic. The position of the intron in the steroid hormone receptors (SH) and the retinoic acid receptors (RAR) and thyroid hormone receptors (TR) are indicated. (D) Alignment of ES43 with members of the nuclear hormone receptor family. Amino acid residues homologous to ES43 are shown in bold. Amino acids, homologous or conserved by hydrophobicity or charge, between ES43 and the receptors are boxed. For conservation comparisons, the following groups of residues have been used: P,A,G,S,T (neutral or weakly hydrophobic); Q,N,E,D (hydrophilic, acid amine); H,K,R (hydrophilic, basic); L,I,V,M,F,Y,W (hydrophobic). Estrogen receptor (ER), Retinoic acid receptor (RAR), Vitamin D (Vit D), Thyroid hormone receptor (TR), Ecdysone receptor (EcR), Glucocorticoid receptor (GR). (E) Alignment of ES43 with the most closely related members of the nuclear receptor family. Amino acid residues homologous to ES43 are shown in bold. Amino acids, homologous or conserved by hydrophobicity or charge, between ES43 and the receptors are boxed. (F) Helical wheel representation of the potential α-helix in the zinc finger of ES43 (left) and the α-helix of the estrogen receptor (right). Amino acid residues are marked according to their position in Fig. 2B and C, respectively. Amino acids in the α-helix of ES43 with positional homology to the DNA-binding amino acid residues of the ER, are marked with identical symbols. Hydrophobic residues are boxed. (G) Alignment of ES43 with part of the most conserved region of the ligand-binding domain of the retinoic acid receptors. Homologous amino acid residues are shown in bold. Amino acid residues, homologous, or conserved by hydrophobicity or charge, between ES43 and the receptors are boxed.

tential secondary structure of the protein was derived using the algorithm of Garnier et al. [18]. The structure is alternated by β-strands, turns and α-helices. The 5′ and 3′ regions are highly hydrophilic, while the center is mainly hydrophobic.

### 3.2. Structure of the potential zinc finger motif in ES43

The potential zinc finger motif of ES43 is defined by four cysteine metal ligands, arranged as a Cys-$X_2$-Cys-$X_{23}$-Cys-$X_2$-Cys ($C_2$–$C_2$) motif. This arrangement is homologous to both zinc finger motifs found in the steroid receptors (Fig. 2C). However, the loop of 23 amino acids between $C_2$ and $C_2$ in ES43 is longer than the 13 amino acids usually found in the loop of the steroid receptor zinc finger motifs. Loops of 20–30 amino acids are found in the putative zinc fingers of the mammalian poly(ADP)-ribose polymerase [19] and uvrA from *E. coli* [20].

Other zinc-finger configurations in ES43 are possible between amino acids $Cys^{166}$ and $His^{171}$ and between $Cys^{190}$ and $Cys^{193}$ resulting in a Cys-$X_4$-His-$X_{17}$-Cys-$X_2$-Cys arrangement or between $His^{171}$ and $Cys^{173}$ and between $Cys^{190}$ and $Cys^{193}$ giving a His-$X_2$-Cys-$X_{14}$-Cys-$X_2$-Cys arrangement. Neither of these arrangements has yet been reported, although Cys-$X_2$-Cys-$X_n$-His-$X_n$-Cys type fingers exist [21].

### 3.3. Homology of ES43 with steroid hormone receptors

ES43 shows homology with the DNA-binding domain of all members of the nuclear hormone receptor family (Fig. 2D). Amino acids homologous or conserved with respect to hydrophobicity and charge, between ES43 and the receptors are boxed in Fig. 2D and E. Three amino acids, $Glu^{25}$, $Gly^{26}$ and $Ala^{29}$ (see Fig. 2C for numbering) determine the specific binding of the estrogen receptor to its DNA response element [6,7]. These three amino acids are present in ES43 at positions $Glu^{191}$, $Gly^{192}$ and $Ala^{195}$ and are positioned in the zinc finger motif equal to the estrogen receptor.

Homologies with several receptors also concern the amino acids $Asp^{168}$ and $Gly^{176}$ positioned in the N-terminal part of the zinc finger, and other residues in the C-terminal part of the finger. In addition, a region of basic amino acids present in the loop of the C-terminal Zn-finger of all receptors, and involved in non-specific DNA-binding, is also present in ES43 ($K^{223}$–$R^{227}$). This short stretch of very basic amino acids might also be functional as a nuclear localization signal (NLS) of the ES43 protein [22]. Homologies are also evident with the genes *egon*, *knirps* and *seven-up* which belong to the nuclear hormone receptor superfamily, but are as yet without known ligand (Fig. 2E) [23]. The homologies extend to amino acids independent from the oligonucleotide used for screening and relate to amino acids $M^{160}$, $Q^{162}$ and $F^{188}$.

### 3.4. Discrepancies between ES43 and steroid hormone receptors

A discrepancy is the addition of 10 amino acids in the loop of the zinc finger. These additional amino acids are part of a potential α-helix between amino acid residues 178 and 193, according to the algorithm of Garnier et al. [18] (Fig. 2F, left), and shows strong homology to the α-helix of the estrogen receptor (Fig. 2F, right). The amino acids, $E^{25}$, $G^{26}$, $K^{28}$, $K^{32}$ and $Q^{36}$, responsible for specific binding to the ERE, correspond exactly in their mutual spacing with, respectively, $E^{191}$, $G^{192}$, $N^{187}$, $K^{184}$ and $K^{180}$ of ES43. Interestingly, this additional region shows a strong homology with part of the most conserved region of the ligand binding domain of the retinoic acid receptors (Fig. 2G) which is, however, always located C-terminally to the DNA-binding domain [4]. The DNA recognition helices of the estrogen and glucocorticoid receptors are located in a 3′ position with respect to the first zinc-finger between amino acids 24 and 36 [6,24]. ES43 has no clear α-helix at this position. The position of the α-helix in ES43 shows homology to the position of the helix found in $C_2$–$H_2$ zinc-fingers, where the two histidines are located in the recognition helix, which is further extended into the loop [25,26].

ES43 contains only one putative zinc finger, whereas in the steroid hormone receptor family two zinc fingers are present. In the steroid hormone receptors, the first zinc finger is responsible for specific DNA-binding and the second for protein-protein (dimer) interactions. Amino acid residues responsible for dimerization are present in

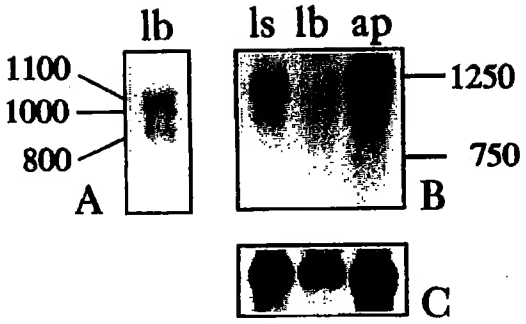


Fig. 3. Expression of ES43 in different tissues. (A) Northern blot containing 2 μg of poly(A)$^+$RNA from leaf blades (lb). (B) Northern blot containing 10 μg total RNA from leaf sheaths, leaf blades and young apices (ap) of the barley dwarf mutant. Filters were probed with labelled ES43 insert. (C) Fragment (panel shows 18-S RNA) of the gel used in (B) stained with methylene blue-as a control for loading.

the so-called D-loop, between $Cys^{43}$ and $Cys^{49}$ (Fig. 2C). Similarities between ES43 and the D-loop of the ER are present (Fig. 2D).

### *3.5. Southern and Northern analyses*

Southern blot analysis showed that the sequence of ES43 is represented by not less than four copies in the barley genome (data not shown). The retinoic acid receptors are encoded by at least three genes. The thyroid hormone receptors by at least two. The steroid hormone receptors are encoded by single copy genes [2].

Northern blot analysis of poly(A)$^+$RNA from leaf blades showed the expression of low-abundant transcripts varying in size between 800 and 1100 nucleotides (Fig. 3a). Several distinct transcripts varying in size between 750 and 1250 nucleotides were also detected in total RNA of leaf sheaths, leaf blades and young apices (Fig. 3b).

For the retinoic acid, estrogen, ecdysone and vitamin D receptors, more than one transcript is expressed by the same gene [2,27]. These isoforms are assembled by alternative promoter usage, and for the retinoic acid- and thyroid hormone receptors, also by differential splicing.

## 4. Discussion

The cDNA clone described, contains a domain very homologous to the DNA-binding domain of the nuclear hormone receptor family. Homologies were found with all members of this family, and were not limited to the sequence of the oligonucleotide used for screening. This is, to our knowledge, the first report on homologies between plant genes and the nuclear hormone receptor family. In addition, no plant homologs of ES43 were found in the *Arabidopsis* and protein databases.

The ES43 protein contains one putative zinc finger, in contrast to the steroid receptors, which always contain two zinc fingers. In the steroid hormone receptors, the two zinc fingers have different functions. The N-terminal finger is involved in specific DNA-binding and contains sufficient information to determine target specificity among the hormone response elements in vivo [28]. The strong homology between the recognition helix of the steroid receptors, and the α-helix of ES43 and particularly the identities in the mutual spacing between the DNA-binding amino acids of the ER and corresponding amino acids in ES43, suggests that ES43 should be sterically capable to bind to an ERE or a derivative.

Homologies with the ligand binding domain of the steroid hormone receptors were, besides of a short sequence in the zinc-finger motif of ES43, not evident. Considering its low conservation among all nuclear receptors, the ligand binding domain was not expected to be present in ES43 in its usual structure.

The ES43 protein contains a repeat of cysteine residues. Cysteine rich repeats that function as ligand binding domains are found in several receptor proteins not related to the steroid hormone receptors, i.e. the LDL (low density lipoprotein) receptor [29], the EGF receptor [30], the insulin receptor [31] and other members of the tyrosine protein kinase family. The cysteine repeat of ES43 could therefore be considered as a putative target for ligand binding studies.

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